An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors

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We recently reported identification of a previously undescribed gammaretrovirus genome, xenotropic murine leukemia virus-related virus (XMRV), in prostate cancer tissue from patients homozygous for a reduced activity variant of the antiviral enzyme RNase L. Here we constructed a full-length XMRV genome from prostate tissue RNA and showed that the molecular viral clone is replication-competent. XMRV replication in the prostate cancer cell line DU145 was sensitive to inhibition by IFN-β. However, LNCaP prostate cancer cells, which are deficient in JAK1 and RNase L, were resistant to the effects of IFN-β against XMRV. Furthermore, DU145 cells rendered deficient in RNase L with siRNA were partially resistant to IFN inhibition of XMRV. Expression in hamster cells of the xenotropic and polytropic retrovirus receptor 1 allowed these cells to be infected by XMRV. XMRV provirus integration sites were mapped in DNA isolated from human prostate tumor tissue to genes for two transcription factors (NFATc3 and CREB5) and to a gene encoding a suppressor of androgen receptor transactivation (APPBP2/PAT1/ARA67). Our studies demonstrate that XMRV is a virus that has infected humans and is susceptible to inhibition by IFN and its downstream effector, RNase L.

cancer | RNase L | xenotropic murine leukemia virus-related virus

A diverse range of mammalian species are susceptible to infections by viruses from the gammaretrovirus genus of *Retroviridae* (1). Examples of these simple viruses whose genomes include *gag*, *pro*, *pol*, and *env* genes only are murine leukemia virus (MLV), feline leukemia virus, koala retrovirus, and gibbon ape leukemia virus. These viruses are responsible for leukemogenesis and other diseases in their respective host species (1–3). However, until recently evidence of authentic infections of humans by gammaretroviruses was lacking. We reported in 2006 identification of viral genomes for a previously undescribed gammaretrovirus, termed xenotropic MLV-related virus (XMRV), in a subset of men with prostate cancer (4). The discovery of XMRV followed investigations of the role of the antiviral enzyme RNase L in hereditary prostate cancer, a disease in which tumors arise in three or more first-degree relatives (5). The human RNase L gene (*RNASEL*) was initially proposed as a candidate for the hereditary prostate cancer 1 (*HPC1*) gene based on a positional cloning/candidate gene method (6).

RNase L is a regulated endoribonuclease for single-stranded RNA that functions in the IFN antiviral response (7, 8). IFN treatment of cells induces a family of 2′–5′ oligoadenylate synthetases that produce 5′-phosphorylated, 2′–5′-linked oligoadenylates (2–5A) from ATP in response to stimulation by viral dsRNA. 2–5A activates the preexisting, latent, and ubiquitous RNase L, resulting in degradation of viral and cellular RNA. Sustained activation of RNase L leads to apoptosis, a function consistent with a role in the suppression of tumor growth (9). Although mice lacking RNase L do not spontaneously develop tumors at higher rates than wild-type mice (our unpublished observations), they are highly susceptible to viral infections (10–12). Therefore, the antitumor effect of RNase L could be a result of the elimination of viral infections involved in cancer etiology.

The suggestion that an antiviral gene suppresses hereditary prostate cancer led us to previously examine the possibility that chronic viral infection might be one factor that, directly or indirectly, predisposes men to prostate cancer (4). Remarkably, XMRV was present almost exclusively in men who were homozygous for a reduced activity variant of RNase L (R462Q). XMRV nucleic acid and Gag protein were found in a small proportion of prostatic stromal cells and fibroblastic and hematopoietic elements, but not in epithelial cells. Here we report that XMRV cDNA derived from human prostate tissue is infectious and that RNase L is required for a complete IFN antiviral response against XMRV. Furthermore, we have affirmed that the likely receptor, xenotropic and polytropic retrovirus receptor 1 (XPR1), is required for XMRV infection, and we have determined the first integration sites of XMRV in human genomic DNA isolated from tumor-bearing prostatic tissue, thus validating that humans have been infected with this virus.

Results

Construction of a Full-Length, Replication-Competent XMRV Clone. To generate a full-length XMRV molecular viral clone, two overlapping partial cDNAs of XMRV strain VP62 were joined after extending the 5′ terminus of cDNA, AO-H4, beyond a unique SalI site (Fig. 1*A* and *Materials and Methods*) (4). Complete sequencing of the full-length XMRV VP62 was performed to validate the clone (GenBank accession no. EF185282). The human prostate cancer cell line LNCaP was chosen to grow XMRV because these cells lack JAK1 required for type I IFN signaling because of an epigenetic silencing of this gene, and because these cells are partially deficient in RNase L because of a deletion in one allele (13, 14). Therefore, we surmised that XMRV would infect and readily replicate in LNCaP cells. The

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Abbreviations: CM, conditioned media; MLV, murine leukemia virus; RT, reverse transcriptase; XMRV, xenotropic MLV-related virus; XPR1, xenotropic and polytropic retrovirus receptor 1; 2–5A, 5′-phosphorylated, 2′–5′-linked oligoadenylates.

Data deposition: The sequence of the molecular viral clone of XMRV VP62 reported in this paper has been deposited in the GenBank database (accession no. EF185282).

See Commentary on page 1449.

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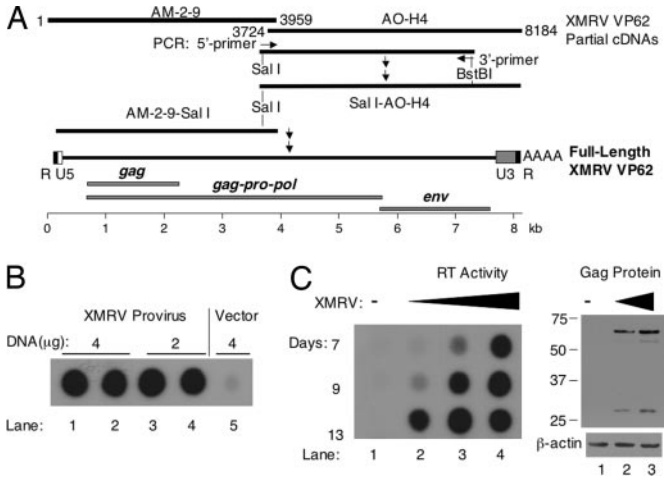

Fig. 1. Cloning of full-length, replication-competent XMRV strain VP62. (*A*) Cloning strategy for assembling complete XMRV molecular viral clone VP62. The cloning diagram is aligned to the gene map. (*B*) Radiolabeled RT products from CM of LNCaP cells transfected for 10 days with 4 μg (lanes 1 and 2) and 2 μg (lanes 3 and 4) of VP62/pcDNA3.1 or from 4 μg of empty vector pcDNA3.1 (lane 5). (*C Left*) RT products in CM from DU145 cells previously exposed to 100 μl of CM from LNCaP cells transfected with pcDNA3.1 (lane 1) or to 1 μl (lane 2), 10 μl (lane 3), and 100 μl (lane 4) of CM from LNCaP cells transfected with VP62/pcDNA3.1. (*C Right*) Western blot for Gag and β-actin from DU145 cells incubated for 9 days with 100 μl of CM from pcDNA3.1-transfected LNCaP cells (lane 1) or CM from VP62/pcDNA3.1-transfected LNCaP cells. Lane 2, 10 μl of CM; lane 3, 100 μl of CM.

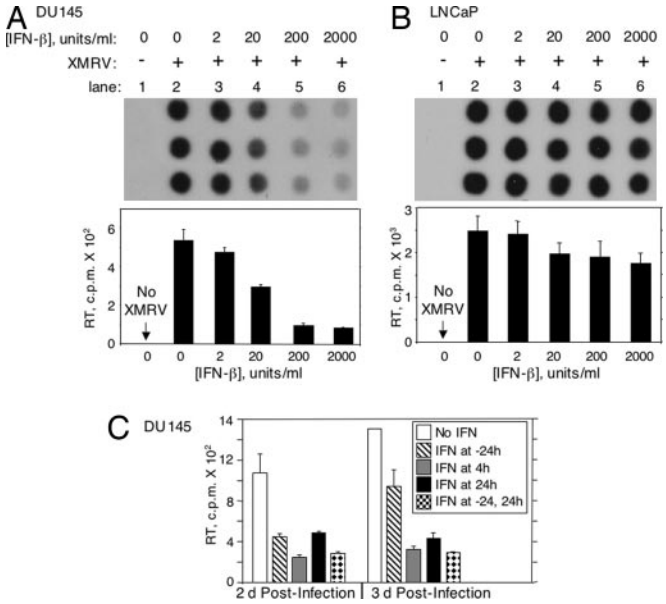

Fig. 2. IFN sensitivity of XMRV in DU145 and LNCaP cells. (*A* and *B*) DU145 cells (*A*) or LNCaP cells (*B*) plated and assayed in triplicate were incubated for 16 h in the absence or presence of different amounts of IFN-β as indicated and then mock-infected (lane 1) or infected with XMRV (lanes 2–6) for 3 days. IFN-β was added a second time at 24 h after infection. (*Upper*) Autoradiograms of the radiolabeled RT products. (*Lower*) RT activity (cpm) as a function of [IFN-β]. (*C*) Effect of time of IFN-β treatment on viral yields in DU145 cells. The IFN added at -24 h only was removed and not replaced at the time of infection. Assays were performed in triplicate. The decreases in RT activity in response to IFN treatments were significant. $P = 0.014$ and 0.018 in two-tailed, paired Student's *t* tests at 20 and 2,000 units/ml IFN-β in *A* and *B*, respectively.

XMRV VP62 clone in vector pcDNA3.1 was transfected into LNCaP cells, and, subsequently, the release of virus particles was monitored by reverse transcriptase (RT) activity in the conditioned media (CM). RT was undetectable in CM from empty vector transfected control cells (Fig. 1*B*, lane 5). However, we observed a high level of RT released from the transfected cells (Fig. 1*B*, lanes 1–4). RT in the CM was first observed at 3 days after transfected with XMRV clone and reached peak amounts after 10 days (Fig. 1*B* and data not shown).

To determine whether the released XMRV particles were infectious, the CM from the transfected LNCaP cells was centrifuged and passed through a 0.2-μm filter before infecting DU145 prostate cancer cells. DU145 cells have wild-type RNase L and are not known to be deficient in IFN signaling (15). XMRV replication, monitored by measuring RT activity in the CM, was clearly detected at 2 days after infection, rising to high levels by 7–13 days after infection (Fig. 1*C Left* and data not shown). There was no RT detectable in the CM of control, uninfected DU145 cells (Fig. 1*C Left*, lane 1). Replication of XMRV was confirmed by detecting Gag protein in Western blots prepared from infected cells at 9 days after infection (Fig. 1*C Right*). We sequenced portions of the *pol* gene from the XMRV produced in both the transfected LNCaP cells and in the infected DU145 cells to verify the identity of the progeny virus as XMRV strain VP62 (data not shown). In addition, XMRV efficiently infected and replicated in both the human ovarian carcinoma cell line Hey1b and the human cervical carcinoma cell line HeLa (data not shown).

XMRV Replication Is Sensitive to Inhibition by IFN-β. To determine the effect of IFN on virus replication, DU145 cells were treated with various doses of human IFN-β and infected with XMRV. At 3 days after infection, RT activity was measured in the CM of the infected and uninfected cells (Fig. 2*A*). The concentration of IFN-β required to reduce viral yields by 50% in DU145 cells was $\approx$20 units/ml. At 200 units/ml IFN, XMRV replication was reduced by $>$80%. In contrast, 2,000 units/ml IFN-β reduced XMRV yields by only $\approx$30% in LNCaP cells (Fig. 2*B*). Even in the absence of added IFN, RT activity was 4-fold higher in CM from XMRV-infected LNCaP cells compared with XMRV-infected DU145 cells (Fig. 2 *A* and *B*).

To investigate whether IFN affected establishment of infection, IFN (200 units/ml) was added to DU145 either before XMRV infections or at various times after infection (Fig. 2*C*). XMRV replication, measured at day 2 or day 3, was similarly inhibited by addition of IFN at 4 or 24 h after infection or both at 24 h before and at 24 h after infection. Addition of IFN 24 h before infection was effective only when measuring RT activity at day 2 but less effective by day 3, suggesting decay of the IFN response pathways by this time point. A similar decay of the IFN response against MLV has been reported (16, 17). Results indicate that IFN inhibited XMRV replication when added either before or after infection.

DU145 Cells Deficient in RNase L Are Partially Resistant to IFN Inhibition of XMRV Replication. To determine the possible involvement of RNase L in the antiviral activity of IFN against XMRV, we used DU145 siRNL cells in which RNase L levels were depressed 2-fold by stable expression of short hairpin RNA against RNase L mRNA (Fig. 3*B Bottom*) (9). For comparison, we used DU145 siRNLm3 cells stably expressing a short hairpin RNA with three mismatched nucleotides. Both cell lines were persistently infected with XMRV for 3 weeks before IFN treatment. In the absence of IFN, XMRV replicated at the same rates in both cell lines as determined by measuring RT activity in CM (Fig. 3*A*). However, at 200 or 2,000 units/ml IFN, the levels of residual virus production in the siRNL cells was approximately twice that of the control, siRNLm3 cells (Fig. 3*B*). Results show an inverse correlation between RT activity in the

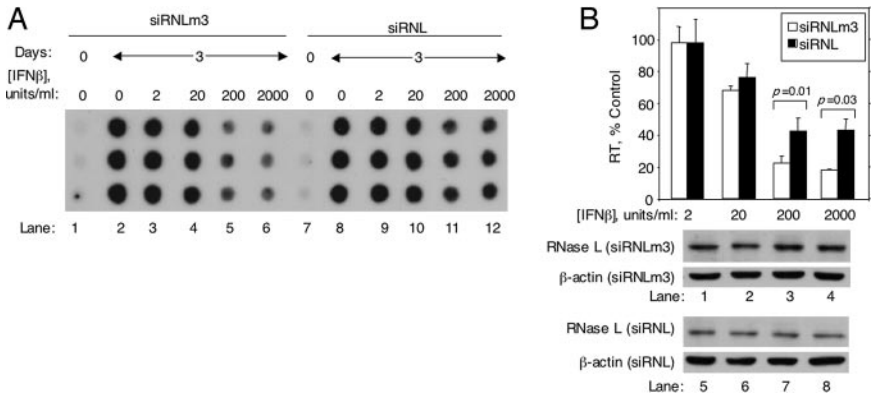

Fig. 3. Effect of RNase L on the antiviral activity of IFN-β. (*A* and *B Top*) RT activities from CM of DU145 cells expressing short hairpin RNA to RNase L (siRNL) or expressing a three-base mismatch control RNA (siRNLm3) (as indicated). Cells were infected for 12 days with XMRV before addition of IFN-β. Lanes 1 and 7, media control; lanes 2–6 and 8–12, CM after 3 days of IFN treatment. (*B Middle* and *Bottom*) Western blots for RNase L and β-actin were from the same experiment, blot, and exposure. Two-tailed, paired Student's *t* tests were performed in *B*.

CM and RNase L in the infected cells, thus implicating RNase L in the IFN antiviral response.

XPR1 Is Required for XMRV Infection. The envelope sequence of XMRV (4) suggests that it is a xenotropic retrovirus; such viruses, which can infect human and other nonrodent cells but not rodent cells, employ XPR1(SYG1) as their entry receptor (4, 18–20). To determine whether XPR1(SYG1) is the receptor for XMRV, human XPR1 cDNA was transiently expressed in nonpermissive hamster CHO cells before exposure to XMRV. A quantitative, real-time RT-PCR method was developed to determine XMRV RNA copy numbers in the CHO cells (Fig. 4*A*). The 5′ primer spanned a 24-bp deletion in the 5′ UTR of *gag* that occurs in all XMRV strains and is a unique and distinctive feature of XMRV (4). As expected, no XMRV RNA was detected in CHO cells that were transfected with empty vector before being exposed to XMRV (Fig. 4*B*). In contrast, in three separate experiments, CHO cells transfected with human XPR1 cDNA before infection with XMRV followed by continued culturing for 30 days resulted in cells that expressed between 20 and 100 copies of XMRV per cell (*Materials and Methods*). To verify these results, RT-PCR was performed for XPR1 mRNA and for *gag* RNA on the same RNA preparations, and the DNA products were analyzed in agarose gels. Similarly, XMRV was present only when CHO cells expressed XPR1 (Fig. 4*C*, lane 4).

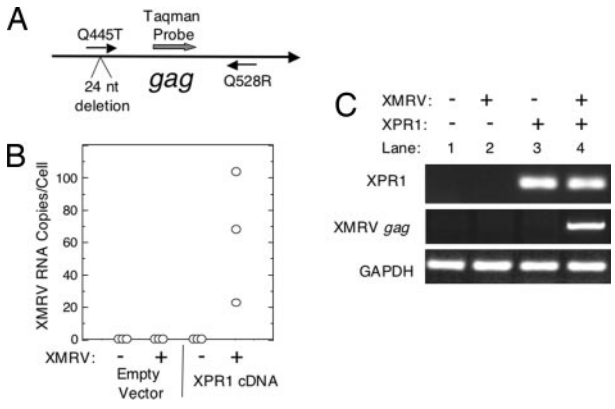

Fig. 4. Infection of hamster cells with XMRV depends on expression of human XPR1. (*A*) Diagram of quantitative real-time RT-PCR strategy for amplifying an 84-bp region from the 5′ UTR of XMRV *gag* (nucleotides 445–528). (*B*) XMRV RNA copy number in CHO cells transiently transfected or mock-infected with empty vector pcDNA3.1 or human XPR1 cDNA in vector pcDNA3.1 followed by exposure to XMRV and continuous culturing for 30 days. The experiment was performed in triplicate. (*C*) Nested RT-PCR for XPR1, XMRV *gag*, and GAPDH RNAs in a representative experiment from *B*. An agarose gel in which the PCR products were stained with ethidium bromide is shown.

XMRV Integration Sites in DNA Isolated from Human Tumor-Bearing Prostatic Tissues. To confirm that XMRV can infect humans, a linker-mediated PCR method was used to map the provirus integration sites in DNA isolated from prostate tissue of men with prostate cancer and germ-line mutations in *RNASEL*. Cases VP234 and VP268 were selected for analysis because both were germ-line homozygous for the R462Q mutation in *RNASEL* and both were XMRV-positive as determined by RT-PCR performed on RNA isolated from prostate tissue (4) (data not shown). We detected XMRV provirus in DNA isolated directly from separate pieces of frozen prostate tissue from both patients. In case VP268, two proviruses were detected from the same prostate tissue sample, one integrated in chromosome 7p15.1 and the other integrated in chromosome 16q22.1 (Fig. 5 *A* and *B*). In both, integration occurred near the transcription start site of genes encoding a transcription factor. In chromosome 7, the XMRV provirus was located 2,640 bp upstream of the transcription start site of cAMP response element-binding protein 5 gene (*CREB5*) (21, 22), and the provirus found in chromosome 16 was located 1,816 bp downstream of the nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3 gene (*NFATc3*) (23–27) transcription start site. These findings are consistent with genome-wide analysis of MLV integration sites indicating that MLV favors transcription units and integrates preferentially near the start of transcriptional units (28, 29). In DNA isolated from prostate tissue of case VP234 we identified in chromosome 17q23.2 the presence of XMRV provirus, which was 11,888 bp downstream of the transcription start site in the amyloid β precursor protein-binding protein 2 (*APPBP2/PAT1/ARA67*) gene (30), encoding a repressor of androgen receptor transactivation (Fig. 5*C*) (31, 32).

Discussion

XMRV Is an Infectious Virus That Replicates Efficiently in Human Prostate Carcinoma Cell Lines. Here we demonstrate that a complete molecular viral clone of XMRV produces an infectious, replication-competent virus. The ability to cultivate XMRV should inform future efforts to understand the association at the cellular and molecular level between infection and pathology. Interestingly, although only stromal cells were observed to contain XMRV nucleic acid or protein in the prostate (4), in cell culture XMRV was

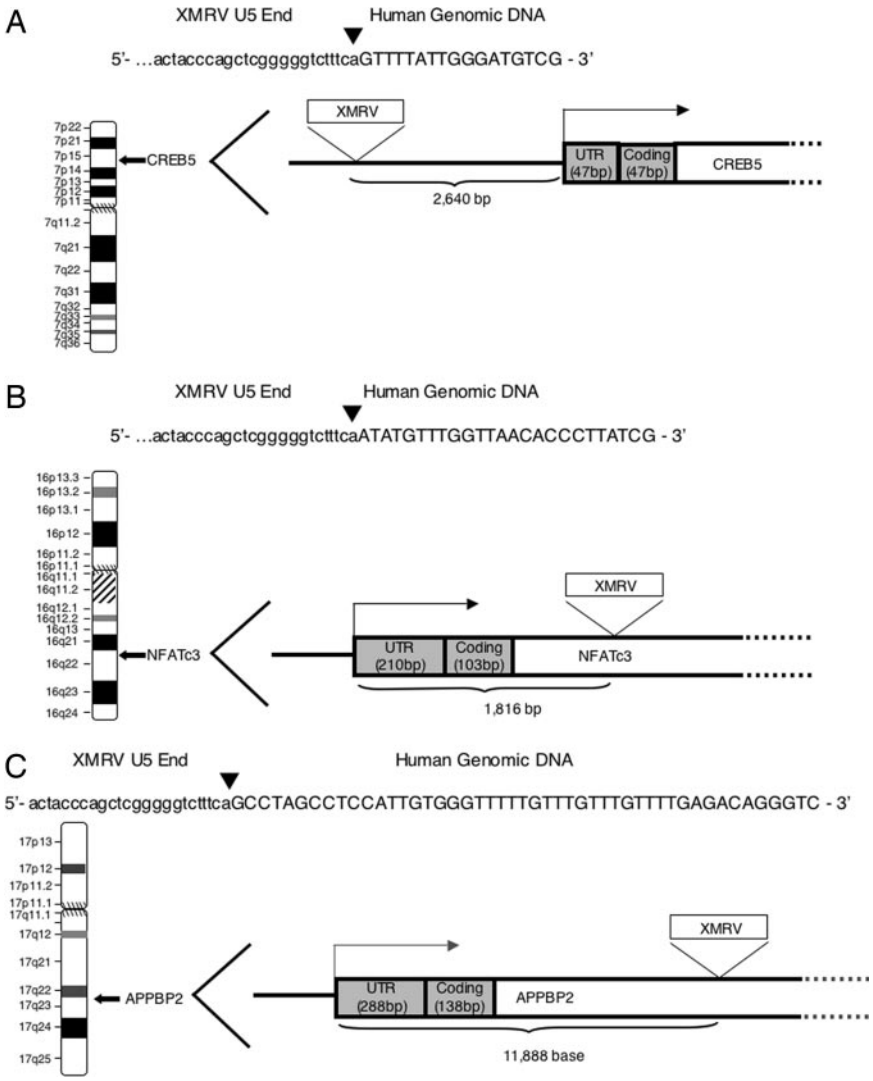

Fig. 5. Locations of XMRV integration sites in prostate DNA from case VP268 (*A* and *B*) and case VP234 (*C*). Genomic DNA was isolated from the patient tumor sample, and the DNA sequence near the virus–host DNA junction was cloned and sequenced (*Materials and Methods*). (*A*) In chromosome 7p15.1 the integrated provirus was 2,640 bp upstream of the *CREB5* transcription start site. (*B*) In chromosome 16q22.1 the integrated provirus was 1,816 bp downstream of the *NFATc3* transcription start site. (*C*) In chromosome 17q23.2 the integrated provirus was 11,888 bp downstream of the *APPBP2* transcription start site. Lowercase letters represent the sequence at the U5 end of the viral long terminal repeat, and uppercase letters represent human genomic sequences. Arrows denote the virus–host DNA junctions. Right-angled arrows denote the transcription start sites.

able to infect and efficiently replicate in two prostate cancer cell lines (DU145 and LNCaP), an ovarian carcinoma cell line (Hey1b), and a cervical carcinoma cell line (HeLa) (Figs. 1 and 2 and data not shown). Perhaps these cell lines, from metastatic or aggressive cancers, have undergone genetic alterations or adaptations in culture that render them susceptible to XMRV; alternatively, it may simply be that XMRV has a wider cell tropism in culture than *in vivo*, a not infrequent finding in other animal viruses. It is possible, even likely, that XMRV infection occurred years before prostate cancer, in which case some cell lineages that were virus-infected could have been eliminated through the host innate or adaptive immune system. The observations of XMRV in prostatic stromal cells *in vivo* could be an index of prior infection and may be distinct from an acute infection. These apparent discrepancies could also be explained by differences in the multiplicity of infection (perhaps much higher *in vitro* than *in vivo*), differences in expression of XPR1 in the cell lines as compared with prostate epithelium, and enhanced replication of XMRV in rapidly dividing cell lines as compared with more slowly dividing cells *in vivo*. At a minimum, our *in vitro* observations suggest that XMRV is at least capable of infecting epithelium under appropriate conditions.

RNase L Is Required for a Complete IFN Antiviral Response Against XMRV. XMRV was identified based on its prevalence in prostate cancer cases with a homozygous mutation in the IFN pathway gene encoding RNase L (R462Q). Therefore, one of the goals of this study was to evaluate the effect of IFN and RNase L on the XMRV life cycle. JAK1-negative LNCaP cells are deficient in IFN signaling, and they also have an inactivating deletion mutation in one allele of *RNASEL* (14). Accordingly, in LNCaP cells IFN-β has only a modest effect on XMRV, and, in the absence of IFN, the LNCaP cells produce several-fold higher levels of XMRV than DU145 cells. In contrast, XMRV was highly susceptible to inhibition by IFN-β in DU145 cells that contain wild-type RNase L (15). IFN was effective against both chronic and acute XMRV infections in DU145 cells (Figs. 2 and 3).

We provide evidence that RNase L is involved in the inhibition of XMRV replication in response to IFN-β treatment. Accord-

ingly, down-regulation of RNase L levels with siRNA rendered DU145 cells partially resistant to the IFN effect against XMRV. In addition, overexpression of a nuclease-dead mutant RNase L (R667A) reduced the IFN effect against XMRV in HeLa cells (data not shown). These findings are entirely consistent with the preferential occurrence of XMRV in prostates from men who are homozygous for the R462Q reduced-activity variant of RNase L (4). How the 2–5A/RNase L pathway suppresses XMRV is unknown. Perhaps highly structured regions in XMRV RNA stimulates 2′–5′ oligoadenylate synthetase to produce 2–5A, which activates RNase L. The activated RNase L would then presumably cleave single-stranded loop regions of XMRV RNA, thus inhibiting virus yields. Our preliminary findings show that some XMRV RNA segments activate 2′–5′ oligoadenylate synthetase *in vitro* (S.H., R. J. Molinaro, and R.H.S., unpublished observations). However, because there are multiple mechanisms for the antiviral action of IFNs (33), there are probably additional IFN pathways capable of suppressing XMRV. For instance, previous studies on MLV and the lentivirus HIV-1 showed that IFN inhibited viral assembly or release (16, 17, 34, 35), a process that has not been shown to be affected by RNase L. Our future efforts will address the stage(s) in the replication cycle of XMRV that are suppressed by IFN.

XPR1 Expression Renders Hamster Cells Susceptible to XMRV Infection. The *env* ORF of gammaretroviruses, including XMRV, are transcribed as a spliced mRNA encoding a precursor of the envelope glycoproteins, the surface subunit (SU), and the transmembrane subunit (1). The transmembrane subunit contains the transmembrane and the hydrophobic fusion segments that function in the fusion of viral and cellular membranes. SU is the major determinant of host range and the receptor-binding site (36). In the SU protein, variable region A functions in receptor recognition and variable region B stabilizes the virus with its specific receptor (36, 37). The variable regions are nearly identical in XMRV and in xenotropic MLV strains, such as DG-75, and are distinct from those in amphotropic and ecotropic MLVs (4, 38). The human cell-surface receptor for xenotropic MLV strains is XPR1, containing multiple transmembrane-spanning domains (18–20, 36). Our results show that expression of human XPR1 in CHO cells was required for XMRV infection of these cells. Despite infection, however, the infected CHO cells produced only low levels of XMRV RNA (20–100 copies per cell as determined by real-time RT-PCR). In contrast, XMRV-infected LNCaP cells contain $\approx 10^6$ XMRV RNA copies per cell (data not shown). The very low levels of XMRV RNA and the fact that 2–4 weeks of culturing was necessary to detect infection indicate that virus replication was severely impeded in CHO cells, probably because of host restriction factors (39). Nevertheless, infection of CHO cells was observed only in the presence of XPR1. Our findings thus show that XPR1 is a cofactor of infection and possibly functions as an XMRV receptor.

Mapping of XMRV Integration Sites in Human Prostate DNA Validates XMRV as a Bona Fide Infection of Humans. The following criteria were used to verify the authenticity of the integration site sequences that we determined in human prostate DNA: (*i*) the sequence contained both right long terminal repeat and linker sequence, (*ii*) a match to the human genome started after the end of the right long terminal repeat (5′-. . . CA-3′) and ended with the linker sequence, and (*iii*) the host DNA region from the putative integration site sequence showed 98% or greater identity to the human genomic sequence. Three provirus integration sites in DNA isolated directly from primary prostate tissues are reported in this study: *CREB5*, *NFATc3*, and *APPBP2*. CREB5, a member of the CRE (cAMP response element)-binding protein family, specifically binds to CRE as a homodimer or a heterodimer with c-Jun or CRE-BP1 and functions as a CRE-dependent transactivator (21, 22). NFATc3 is a member of the nuclear factors of activated T cells DNA-binding transcription complex and plays a role in the regulation of gene expression in T cells and immature thymocytes (23–27). Interestingly, NFATc3 is a site of integration of the SL3-3 murine lymphomagenic retrovirus in mice in which integration represses NFATc3 expression in lymphomas (40). NFATc3-deficient mice infected with SL3-3 virus develop T cell lymphomas with increased frequencies compared with wild-type mice. APPBP2 has homology to the molecular motor protein, kinesin light chain, involved in transport of proteins along microtubules (30). APPBP2, which also binds microtubules, is expressed in a wide range of cell types and functions in the trafficking of amyloid precursor protein (30, 41). Remarkably, APPBP2 also interacts with the androgen receptor and suppresses androgen signaling (31, 32). At present we do not know whether these XMRV integration events affect gene expression and function of these factors or whether the integrations have direct or indirect effects on the etiology or progression of prostate cancer. However, our findings of integration sites for XMRV in human prostate DNA validate that bona fide, naturally occurring XMRV infections of humans have occurred among a subset of prostate cancer cases.

Materials and Methods

Cloning of Full-Length XMRV Strain VP62. Two partially overlapping cDNAs (AM2-9 and AO-H4) of XMRV, cloned from prostate RNA of case VP62, were fused to generate full-length molecular viral clone (Fig. 1*A*) (4). The AO-H4 cDNA was extended in the 5′ direction past a unique SalI site by PCR with a 5′ 73-nt primer from nucleotide 3682 to nucleotide 3754 and a 3′ primer from nucleotide 7474 to nucleotide 7453 using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The PCR product was subcloned into pCR-Blunt-II-TOPO vector (Invitrogen, Carlsbad, CA) and excised with NotI (site in the vector) and BstBI (site in the cDNA) before subcloning into AO-H4 cDNA to produce SalI–AO-H4. The 5′ half of VP62, AM2-9, was subcloned together with the 3′ half clone with SalI and NotI. Finally, the full length of VP62 in pCR2.1 was subcloned into pcDNA3.1(−) vector with NotI and HindIII. To validate the molecular viral clone, it was completely sequenced (GenBank accession no. EF185282).

Cell Lines, XMRV Transfections, Infections, and Treatment with IFN-β. LNCaP-FGC and DU145 cell lines (both from American Type Culture Collection, Manassas, VA) were cultured in RPMI medium 1640 supplemented with 10% FBS. DU145 cell derivatives expressing siRNA to RNase L (siRNL cells) and a control cell line expressing the same siRNA with three mismatched nucleotides and compensatory base changes in the opposite strand to maintain base pairing (siRNLm3 cells) were generated as previously described except the short hairpin RNAs were cloned in pSilencer 3.1–H1 puromycin vector (Ambion, Austin, TX) (9). The clones were selected by using 1 μg/ml puromycin in RPMI medium 1640 supplemented with 10% FBS and penicillin–streptomycin. Plasmid VP62/pcDNA3.1(−) or empty vector was transfected into LNCaP cells with Lipofectamine 2000 (Invitrogen). Beginning after 3 days of transfection the culture medium was centrifuged at 12,000 $\times$ *g* for 5 min and RT activity was measured in the supernatants. To isolate virus, the cell culture supernatants at 10 or 13 days after transfection were passed through a 0.2-μm filter (Whatman, Florham Park, NJ) and stored at −70°C. Virus infections were performed in triplicate in 12-well or six-well plates by using cells plated 1 day before infection. Cells were at ≈50% confluency at the time of infection. The cell media were replaced with media containing 8 μg/ml polybrene and 50 or 100 μl of virus stock and incubated for 3 h to allow virus adsorption. Cells were washed once with PBS, and fresh media containing FBS were then added to the cells. CM from infected cells were used for RT assays, and cell extracts prepared by lysing cells [in 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris·HCl (pH 8.0), and 10 μg/ml leupeptin] were used in Western blots for

detecting Gag protein. Human recombinant IFN-β ($200–400 \times 10^6$ units/mg; InterPharm Laboratories, a gift from Ares-Serono, Rockland, MA) was added to the cell culture media at the times indicated in Figs. 2 and 3. Western blot assays for Gag, RNase L, and β-actin are described in supporting information (SI) *Materials and Methods*.

RT Assays. RT activity was measured by using poly(rA) as template and oligo(dT) as primer as described (42). CM (5 μl) was incubated with 20 μl of 1.2× mixture containing 60 mM Tris·HCl (pH 8.3), 0.7 mM $MnCl_2$, 75 mM NaCl, 0.6% Nonidet P-40, 6 μg/ml poly(dT), 12 μg/ml poly(rA), 12 μM dTTP, 24 μM DTT, and [α-^{32}P]dTTP (10 μCi/ml; 3,000 Ci/mmol) at 37°C for 2 h. Reactions (5 μl) were spotted on DEAE paper (Whatman) followed by washing three times in 0.3 M NaCl/0.03 M sodium citrate (pH 7.0) and briefly rinsing twice with 95% ethanol. Autoradiograms were prepared by exposing x-ray film to the dried paper. The radiolabeled reaction products from the DEAE paper were excised, and the radioactivity (expressed as cpm) for each reaction was determined by liquid scintillation counting.

XPR1 Expression and XMRV Infection of CHO Cells. CHO cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% FBS and nonessential amino acids and plated in triplicate in six-well plates. Human XPR1 cDNA (kindly provided by A. D. Miller, Fred Hutchinson Cancer Research Institute, Seattle, WA) (18) was subcloned into pcDNA 3.1/Zeo(+). The XPR1 cDNA and empty vector control pcDNA 3.1/Zeo(+) were transfected into CHO cells with Lipofectamine 2000. After 24 h the transfected cells were infected with XMRV diluted in DMEM supplemented with 5 μg/ml polybrene without FBS or antibiotics for 3 h after which complete DMEM was added. At 24 h after infection the complete medium was replaced. The infected cells were maintained for up to 30 days. Total RNA was harvested by using TRIzol (Sigma–Aldrich, St. Louis, MO), and cDNA was synthesized with random hexamer by using an iScript select cDNA synthesis kit (Bio-Rad, Hercules, CA) for use in quantitative RT-PCR and nested RT-PCR (described in *SI Materials and Methods*).

Cloning and Sequencing XMRV Integration Sites. DNA was isolated by using QIAamp DNA mini kits (Qiagen, Valencia, CA) from ≈5-mg cores of peripheral-zone prostate tissue (containing tumor cells) after prostatectomy and stored at −80°C. DNA was eluted in 400 μl of 10 mM Tris·Cl/0.5 mM EDTA (pH 8.0). The assay for cloning and sequencing XMRV integration sites was essentially identical to that described previously for HIV-1 (43) and is described in *SI Materials and Methods*.

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